

Identification of a Cellular Repressor of Transcription of the Adenoviral Late IVa₂ Gene That Is Unaltered in Activity in Infected Cells

Huey-Jen L. Lin¹ and S. J. Flint²

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544-1014

Received June 20, 2000; returned to author for revision August 2, 2000; accepted August 18, 2000

The gene encoding the adenovirus type 2 IVa₂ protein, a sequence-specific activator of transcription from the viral major late promoter, is itself transcribed only during the late phase of infection. We previously identified a cellular protein (IVa₂-RF) that binds specifically to an intragenic sequence of the IVa₂ transcription unit. We now report that precise substitutions within the IVa₂-RF-binding site that decreased binding affinity increased the efficiency of IVa₂ transcription in *in vitro* reactions containing IVa₂-RF. Consistent with the conclusion that this cellular protein represses IVa₂ transcription, mutations that led to more efficient transcription in the presence of IVa₂-RF were without effect in reactions lacking this cellular protein. No change in the concentration or activity of IVa₂-RF could be detected in adenovirus-infected cells during the period in which the IVa₂ gene is transcribed. We therefore propose that restriction of IVa₂ transcription to the late phase is the result of titration of this cellular repressor as the number of copies of the IVa₂ promoter increases upon replication of the viral genome. © 2000 Academic Press

Key Words: human adenovirus type 2; IVa₂ promoter; IVa₂ transcription; transcriptional repressor; repressor titration.

INTRODUCTION

A characteristic feature of the replication of human subgroup C adenoviruses is the strict temporal regulation of expression of viral genetic information. Prior to the onset of viral DNA synthesis, nonstructural proteins that are necessary for viral DNA synthesis, that regulate various reactions in the production of viral mRNA, or that counteract host defense mechanism are synthesized (see Shenk, 1996). Proteins encoded within the immediate-early E1A transcription unit, which is transcribed by the cellular RNA polymerase II transcriptional machinery alone, are the first to be expressed in the infected cell and are required for efficient transcription of viral early genes (Berk *et al.*, 1979; Jones and Shenk, 1979; Nevins, 1981; see Berk, 1986; Shenk, 1996). The larger (289R) of the two abundant E1A proteins stimulates transcription of all viral early genes in infected cells (Berk *et al.*, 1979; Jones and Shenk, 1979; Nevins, 1981) and can increase the activity of a great variety of viral and cellular promoters in experimental systems (see Jones, 1995; Shenk and Flint, 1991). The unique internal sequence of this protein, which contains the third of three sequences conserved among human adenoviruses, designated conserved regions 1–3 (CR1–CR3) (Moran and Mathews, 1987), appears to operate via both cellular, sequence-specific

transcriptional regulators (Flint and Jones, 1991; Liu and Green, 1990) and components of the general initiation machinery (Boyer *et al.*, 1999; Chiang and Roeder, 1995; Geisberg *et al.*, 1994, 1995; Lee *et al.*, 1991; Mazzarelli *et al.*, 1997). The CR1 and CR2 sequences present in both E1A proteins also regulate transcription. For example, they mediate binding of E1A proteins to the cellular retinoblastoma protein (Rb) to induce the release of members of the E2F family of transcriptional activators from association with Rb and thus transcription of E2F-dependent genes, including several that are crucial for progression from the G1 to the S phase of the cell cycle (see Bartek *et al.*, 1996; Flint and Shenk, 1997; Nevins, 1992; Weinberg, 1995), and the adenoviral E2 early promoter (Kovesdi *et al.*, 1986a,b; Manohar *et al.*, 1990; Zajchowski *et al.*, 1985). Once E2 replication proteins have accumulated to sufficient concentrations, viral DNA synthesis begins within the infected cell nucleus. This process not only produces the genomes that will be incorporated into progeny virions, but also is crucial for establishment of the transcriptional program characteristic of the late phase of infection (see Flint, 1986; Shenk, 1996 for reviews). Although long recognized, the various viral DNA replication-dependent changes in transcription from the adenoviral genome have received relatively little attention and remain incompletely understood.

During the late phase, the major late (ML) transcription unit is transcribed to sites close to the right-hand end of the linear, double-stranded viral DNA genome (Fraser *et al.*, 1979). In contrast, during the early phase, transcription from the ML promoter terminates at multiple sites

¹ Current address: Department of Pediatrics, Cancer Center, University of Michigan, 1500 E. Medical Center Drive, Ann Arbor, MI 48105.

² To whom correspondence and reprint requests should be addressed. Fax: (609) 258-4575. E-mail: sjflint@molbio.princeton.edu.

between about 40 and 75 map units (Akusjärvi and Persson, 1981; Iwamoto *et al.*, 1986; Nevins, 1981; Shaw and Ziff, 1980). Coinfection experiments established that replication of the viral genome is a prerequisite for complete transcription of the ML transcription unit (Crossland and Raskas, 1983; Thomas and Mathews, 1980). Indeed, the production of replicated viral DNA molecules in the infected cell may be sufficient (Larsson *et al.*, 1992). This requirement strongly suggests that some structural feature of the viral genome, or of the nucleoprotein in which it resides during the early phase of infection, prevents transcription of the promoter-distal portion of the ML transcription unit. However, no direct support for this model has yet been collected. Run-on transcription assays established that some 80% of transcription complexes traversing the ML transcription unit fail to reach the second exon and terminate transcription between positions +190 and +1150 when synthesis of viral late proteins is prevented (Larsson *et al.*, 1992). The viral late protein(s) required for relief from such premature termination of transcription within the first ML intron has not been identified, nor is it known whether replicated viral DNA templates are also necessary.

Entry into the last phase of infection also induces a large increase in the rate of transcription from the ML promoter (Shaw and Ziff, 1980). Such stimulation of ML transcription in infected cells or in extracts prepared from them requires sequences within the first intron (Alonso-Caplen *et al.*, 1988; Jansen-Durr *et al.*, 1988, 1989; Leong *et al.*, 1990; Mansour *et al.*, 1986; Mason *et al.*, 1990). The two intronic sequences primarily responsible for stimulation of ML transcription, designated DE1 (+86 to +96) and DE2 (+113 to +124), are specifically recognized by proteins present in adenovirus-infected cells only during the late phase of infection (Jansen-Durr *et al.*, 1988, 1989; Leong *et al.*, 1990; Mondésert *et al.*, 1992). The protein named DEF-A binds specifically to DE1 and with lower affinity to the 3' portion of the DE2 sequence, whereas the infected cell-specific protein termed DEF-B interacts with the 5' segment of DE2 (Jansen-Durr *et al.*, 1989; Mondésert *et al.*, 1992). Keding and colleagues identified the viral IVa₂ protein as the sole component of DEF-B and demonstrated that DEF-A comprises the IVa₂ protein in association with at least one other infected cell-specific protein (Lutz and Keding, 1996; Tribouley *et al.*, 1994). Thus, in addition to its probable role in assembly (Gustin *et al.*, 1996; Persson *et al.*, 1979; Winter and D'Halluin, 1991; Zhang and Imperiale, 2000), the adenoviral IVa₂ protein is a sequence-specific transcriptional activator. Its function as components of both DEF-A and DEF-B accounts for the late-phase-specific stimulation of ML transcription: IVa₂ mRNA is not synthesized during the early phase of infection (Binger and Flint, 1984; Chow *et al.*, 1979; Crossland and Raskas, 1983; Winter and D'Halluin, 1991); the DNA-binding activities of DEF-A or DEF-B cannot be

detected until the IVa₂ protein is synthesized (Lutz and Keding, 1996), and production of IVa₂ mRNA and protein precedes synthesis of ML mRNAs in the late phase of infection (Binger and Flint, 1984; Winter and D'Halluin, 1991). On the other hand, the IVa₂ promoter is one of three in the adenoviral genome that are recognized only following initiation of viral DNA synthesis (see Flint, 1986; Shenk, 1996). As the activation of IVa₂ transcription appears to be crucial for efficient production of viral structural proteins and successful completion of the infectious cycle, the mechanism by which the activity of this promoter is regulated during productive infection is of considerable interest.

The initiation sites of the adenovirus type 2 (HAV-2) IVa₂ and ML transcription units, which lie on opposite strands of the viral genome, are separated by some 210 bp (Fig. 1). This arrangement of divergent transcription units, in conjunction with results of *in vitro* transcription and DNase I footprinting assays, led to the proposal that the ML and the IVa₂ promoters share binding sites for USF/MLTF and thus compete for this transcriptional regulator (Adami and Babiss, 1992; Carcamo *et al.*, 1989; Moncollin *et al.*, 1990; Natarajan *et al.*, 1984). In principle, such competition could preclude IVa₂ transcription during the early phase of infection, when ML transcription is stimulated by the 289R E1A protein (Leong *et al.*, 1988; Nevins, 1981). However, no increase in the production of IVa₂ mRNA was observed in cells infected by viruses carrying USF/MLTF-binding site mutations or additional mutations that impaired ML transcription (Reach *et al.*, 1990), strongly arguing that competition for this (or any other) cellular protein is not an important mechanism of regulation of the activity of the IVa₂ promoter in infected cells. Although the IVa₂ promoter is not active until the late phase of adenovirus infection, it can be accurately and quite efficiently recognized by the cellular transcriptional machinery under specific *in vitro* conditions (Kasai *et al.*, 1992; Leong and Flint, 1984; Matsui, 1982; Natarajan *et al.*, 1983, 1984). Such transcription requires an autonomous initiator sequence that directs transcription from the major start site observed in infected cells and an intragenic sequence essential for efficient transcription (Carcamo *et al.*, 1991; Chen and Flint, 1992; Kasai *et al.*, 1992). A binding site for a cellular protein centered at position -47 of the transcription unit stimulates IVa₂ transcription *in vitro*, but by only a modest degree (Kasai *et al.*, 1992; Natarajan *et al.*, 1984; Natarajan and Salzman, 1985). The mechanism of recognition of the IVa₂ promoter, which lacks a TATA sequence (Chen and Flint, 1992), has not been elucidated, although all RNA polymerase II general initiation proteins are necessary (Carcamo *et al.*, 1989). In previous studies, we identified a cellular protein that binds specifically to intragenic IVa₂ sequences, making contacts between positions +11 and +27 (Chen *et al.*, 1994). As preliminary analyses suggested that binding of this protein correlated with inhibi-

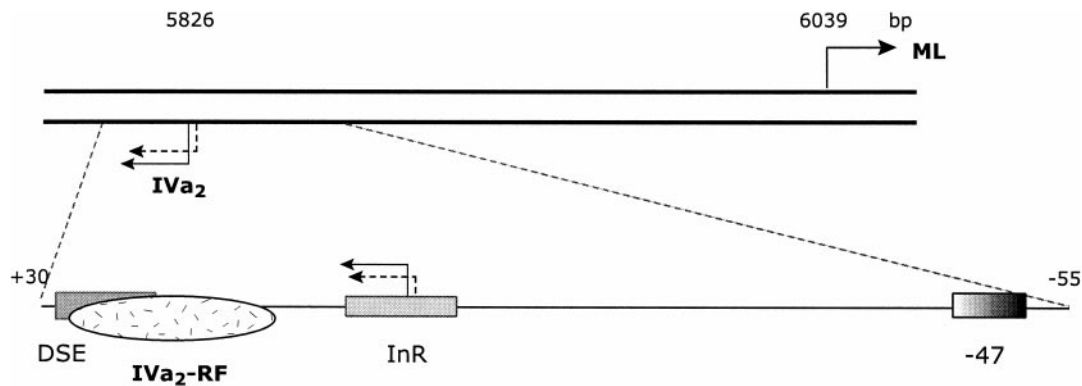


FIG. 1. Organization of the subgroup C adenovirus IVa₂ promoter. The segment of the viral DNA genome containing the 5' ends of the IVa₂ and ML transcription units is represented to scale by the solid, horizontal lines at the top, with sites of transcriptional initiation indicated by arrows drawn in the direction of transcription. The positions of these ML and IVa₂ initiation sites in the genome (bp) are listed at the top. The region containing the IVa₂ promoter, with the major and minor initiation sites shown by solid and dashed arrows, respectively, is expanded at the bottom. The promoter sequences shown, an initiator (InR), the intragenic sequence essential for efficient, initiator-dependent transcription (DSE-s), and an upstream sequence (−47) that stimulates IVa₂ transcription by a factor of 3–5 were identified by mutational analyses using *in vitro* transcription reactions (Chen and Flint, 1992; Chen *et al.*, 1994; Kasai *et al.*, 1992). The oval indicates the intragenic sequence contacted by the cellular protein IVa₂-RF (Chen *et al.*, 1994).

tion of IVa₂ transcription, the protein was designated IVa₂-repressing factor (IVa₂-RF). The presence of a repressor of IVa₂ transcription in HeLa cells could readily account for the inactivity of the promoter during the early phase of infection. We have therefore investigated in more detail the role of this cellular protein in transcription from the IVa₂ promoter and determined the effects of adenovirus infection upon its activity.

RESULTS

Mutations of intragenic IVa₂ promoter sequences that impair binding of a cellular protein

Our previous conclusion that binding of the cellular IVa₂-RF protein to intragenic promoter sequences inhibits IVa₂ transcription (see Introduction) was based on analysis of the effects of a minimal number of promoter mutations. As the IVa₂ sequence from positions −4 and +30 is packed with transcriptional control signals, which appear to overlap (Chen and Flint, 1992; Chen *et al.*, 1994; Fig. 1), a more extensive mutational analysis was initially undertaken to confirm the function of IVa₂-RF in regulation of the activity of the IVa₂ promoter. A series of precise substitutions between positions +10 and +20 of the viral transcription unit was introduced into both plasmid templates for IVa₂ transcription *in vitro* and double-stranded oligonucleotides containing the IVa₂-RF binding site, as described under Materials and Methods. The location of these mutations was chosen to attempt to avoid alteration of the previously identified, downstream sequence essential for efficient initiation of transcription from the IVa₂ initiator element, upon which the binding site for IVa₂-RF is superimposed (Fig. 1; Chen *et al.*, 1994). Another consideration important to the goal of investigating regulation of IVa₂ transcription during pro-

ductive infection was the effects of the substitutions on the coding sequence for the viral DNA polymerase, within which the IVa₂ promoter lies (Fig. 2). The effects of the mutations on recognition of its intragenic binding site by IVa₂-RF were examined using electrophoretic mobility shift assays with wild-type and mutant DNA fragments as competitors. A single complex sensitive to competition by unlabeled IVa₂ DNA, but refractory to competition by an unrelated viral DNA fragment, was observed when the partially purified protein was incubated with a ³²P-labeled DNA fragment comprising position +2 to +35 of the IVa₂ promoter (Fig. 3A, lanes 1–9). The mutations listed in Fig. 2 reduced the ability of unlabeled DNA fragments to compete for such specific binding of partially purified IVa₂-RF to the wild-type IVa₂ sequence (e.g., Fig. 2A, lanes 10–26). The inhibition of binding induced by these substitutions was not surprising, as this region of the IVa₂ transcription unit is contacted on both DNA strands by the cellular protein (Chen *et al.*, 1994). In an attempt to distinguish sequence features important for binding of IVa₂-RF, the effects of the mutations were assessed more quantitatively. The quantities of specific complex formed in the presence of increasing concentrations of each mutant competitor, or of the wild-type sequence, were measured using a phosphorimager. The concentrations of competitor required to inhibit binding to the wild-type IVa₂ DNA fragment by 50% were then determined from plots like those shown in Fig. 3B and used to calculate the affinities with which the cellular protein bound to each mutant DNA relative to that for binding to the wild-type promoter (Fig. 2).

The substitutions could be divided into two classes by the criterion of whether they reduced the affinity of IVa₂-RF for the internal promoter sequence by a factor of 5–10 or were significantly more deleterious (Figs. 2 and

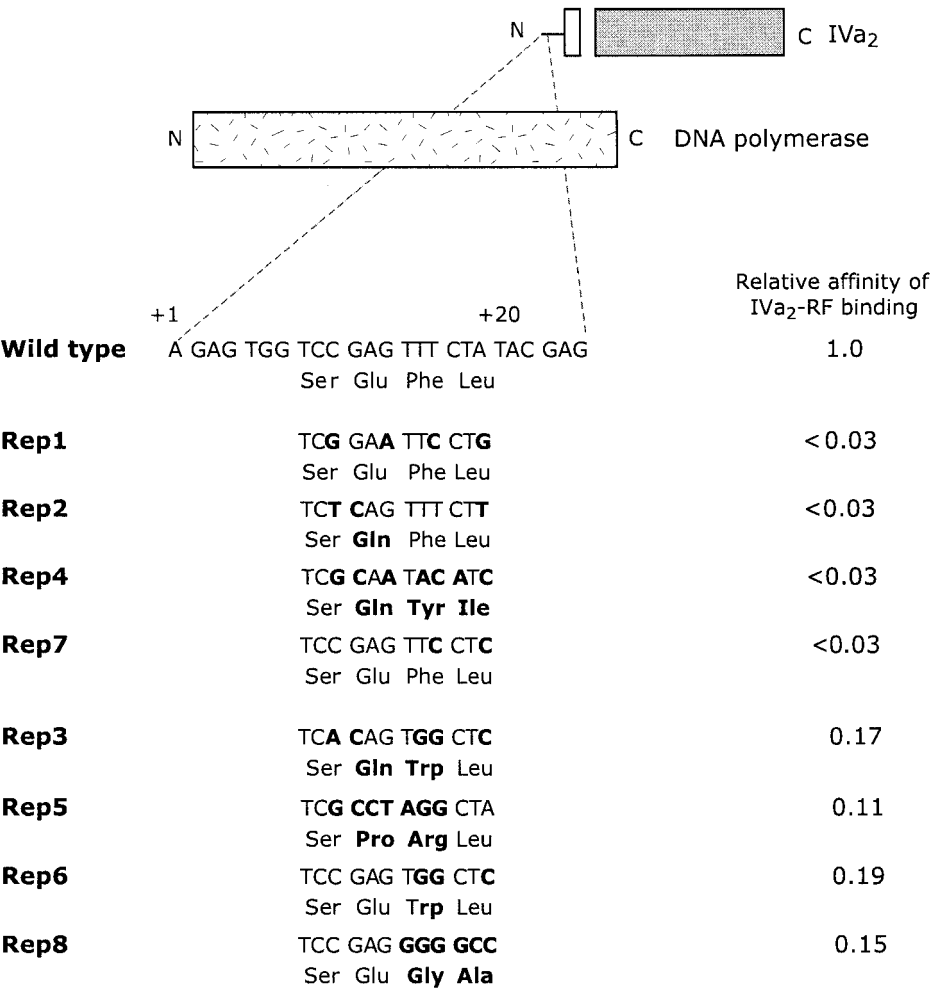


FIG. 2. Design and properties of mutations within the negative regulatory sequence of the IVa₂ promoter. The position of the IVa₂ 5' untranslated sequence, which includes the intragenic promoter sequences shown in Fig. 1, and coding sequence relative to that of the overlapping reading frame encoding the viral DNA polymerase in the I strand of the HAV-2 genome is shown to scale at the top. The sequence of the first 25 bases of the coding strand of the IVa₂ transcription unit is given below, arranged to show the codons of the DNA polymerase reading frame and, for the four central codons of this sequence, the amino acids they specify. The sequences from position +8 to position +19 of 8 precise substitution mutations, Rep1–Rep8, and the changes, if any, introduced into the DNA polymerase coding sequence, are listed below, with altered bases and amino acids shown in boldface type. The effects of these mutations on the affinity with which IVa₂-RF binds to DNA are summarized at the right. The molar excess concentrations of wild-type or mutant IVa₂ DNA fragments required for 50% inhibition of binding of the protein to the wild-type IVa₂ sequence were determined as illustrated in Fig. 3. These values were then used to calculate the affinities of binding of IVa₂-RF to the eight mutant sequences, relative to that of its binding to the wild-type promoter assigned a value of 1.0.

3B). Comparison of the sequences altered in the members of the two classes of mutation indicated that this protein can tolerate some surprisingly extensive changes in its binding site. For example, substitution of the 7 consecutive basepairs occupying positions +10 to +16, as in the Rep5 mutant, reduced binding by only some 10-fold (Fig. 2). Nevertheless, certain basepair substitutions within the IVa₂-RF binding site severely impaired its recognition. Three of four altered sequences to which the protein bound with greatly reduced affinity, Rep1, Rep4, and Rep7, contain a CG pair in place of the TA pair at position +16 of the wild-type sequence (Fig. 2). Because the only other alteration introduced into the Rep7 mutant, substitution of a CG for an AT basepair at

position +19, was also present in mutated sequences to which IVa₂-RF bound relatively well, such as Rep6 (Figs. 2 and 3), we can conclude that introduction of a CG basepair at position +16 severely impairs binding of the protein. Such inhibition of binding does not appear to be the result of loss of a specific base contact, for the A in the noncoding strand at position +16 is not contacted by IVa₂-RF (Chen *et al.*, 1994). However, it is adjacent to an A at position +15 in the noncoding strand, whose methylation enhanced binding of the protein (Chen *et al.*, 1994). Furthermore, a GC rather than a CG basepair at this position, as in Rep6, Rep3, Rep8, and Rep5 DNAs, resulted in a much smaller degree of inhibition of binding, regardless of the nature and number of other sub-

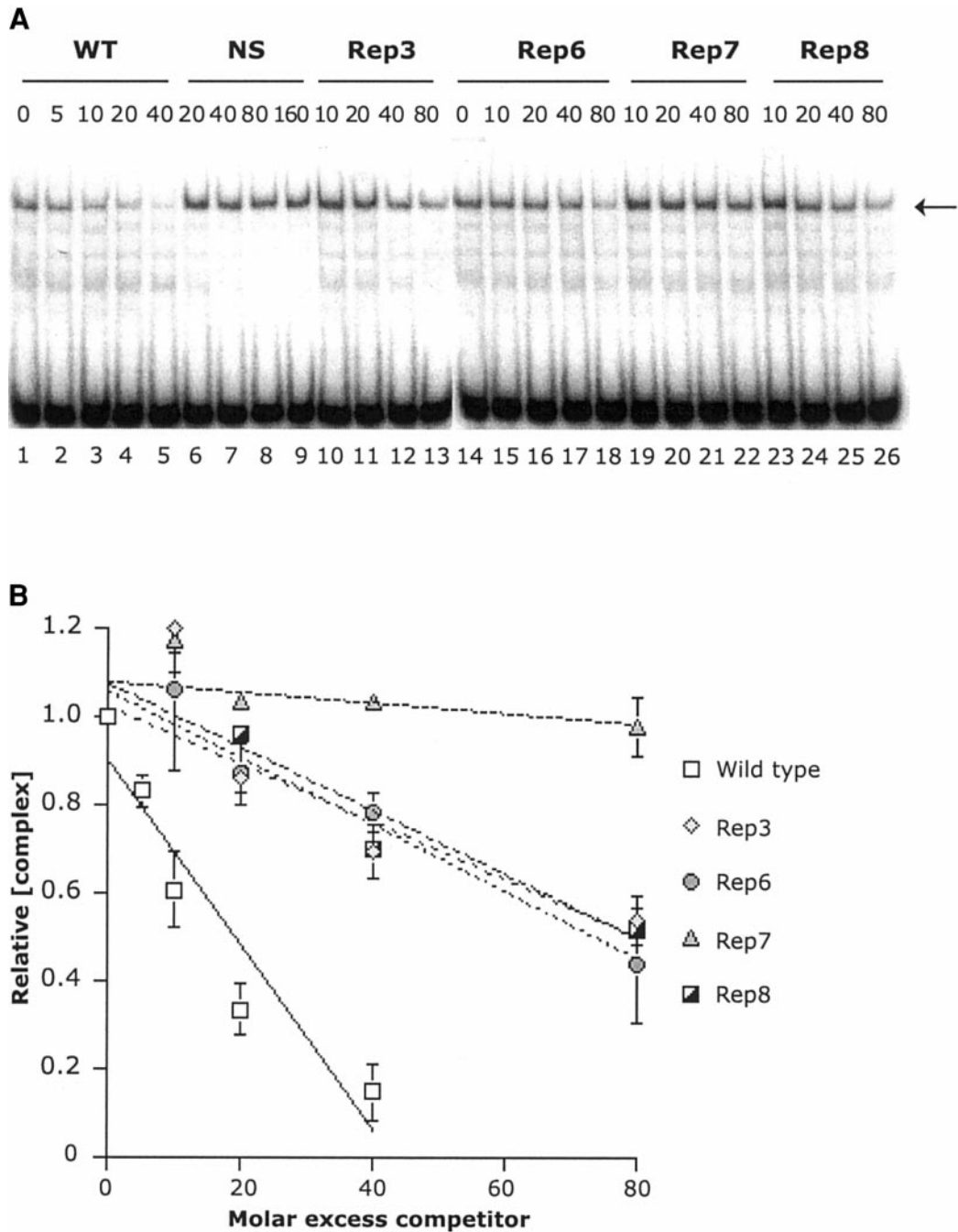


FIG. 3. Intragenic substitutions impair binding of IVa₂-RF to the IVa₂ promoter to different degrees. Electrophoretic mobility shift assays (A) with partially purified IVa₂-RF were performed as described under Materials and Methods. Binding reactions contained no competitor DNA (lane 1) or the molar excess concentrations indicated of the unlabeled wild-type or mutated IVa₂ DNA fragments, or of an unrelated DNA fragment containing a mutated version of the ML sequence +80 to +120 (NS), listed at the top. The specific complex containing IVa₂-RF is indicated by the arrow at the right. Lanes 1–13 and 14–26 represent two gels run in parallel. The mutations present in the Rep3, Rep6, Rep7, and Rep8 DNAs are shown in Fig. 2. Quantification of inhibition of IVa₂-RF binding as a function of competitor concentration is illustrated in B. Each point represents the mean of at least three independent assays.

stitutions present in these mutant DNAs (Figs. 2 and 3). These observations suggest that the conformation of the DNA sequence with which IVa₂-RF interacts, or its conformational deformability, is an important determinant of specific binding of the protein to the IVa₂ promoter. Ad-

ditional experiments will be required to test this possibility more directly, as well as to assess the contributions of individual base contacts made by the protein between positions +11 and +27 (Chen *et al.*, 1994) to the IVa₂-RF-DNA interaction.

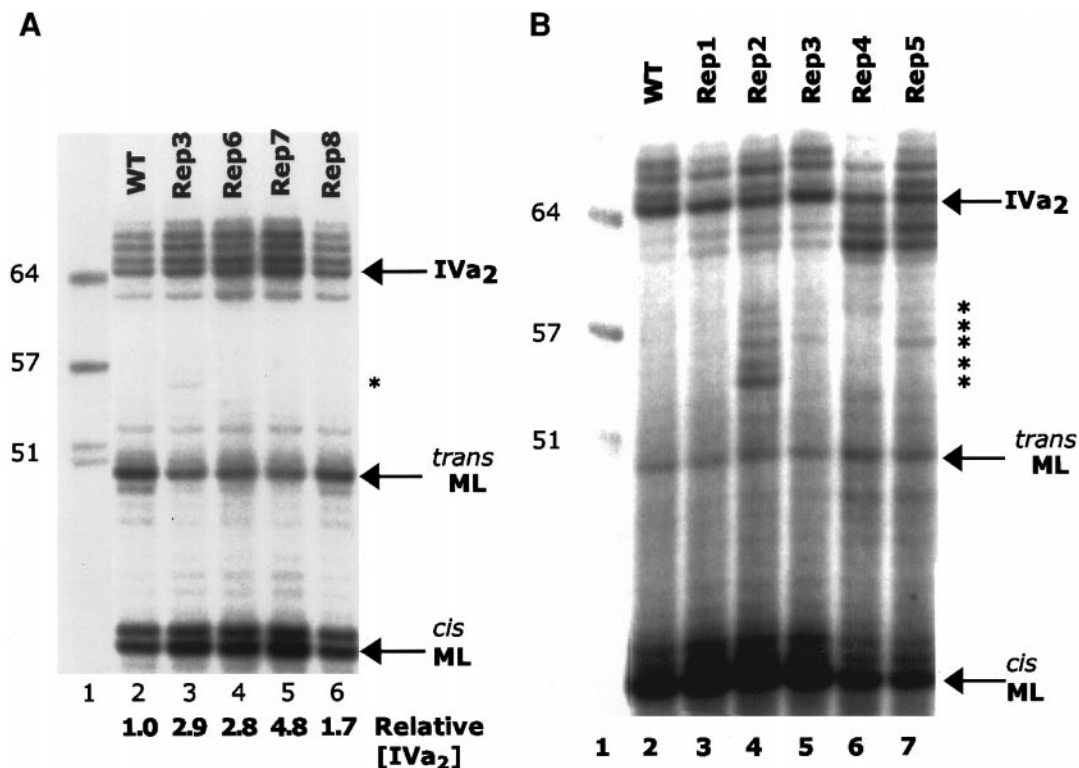


FIG. 4. Alterations in IVa₂ and ML transcription by whole cell extract proteins induced by the Rep mutations. (A) Transcription reactions were as described under Materials and Methods and contained the pML(C₂AT) Δ -50 internal control template and the wild-type pIVa₂-ML template (lane 2) or the Rep3, Rep6, Rep7, or Rep8 mutant templates (lanes 3–6). End-labeled *Hae*III fragments of pBR322 DNA used as markers were loaded in lane 1. The positions of cDNAs copied from IVa₂ and ML transcripts of pIVa₂-ML templates (IVa₂ and *cis*-ML, respectively) and the internal control template (*trans*-ML) are indicated at the right, and the lengths of DNA markers are shown at the left. The relative concentrations of IVa₂ transcripts shown were measured and corrected using the internal control transcript, as described under Materials and Methods. (B) Transcription reactions contained the internal control template and the wild-type pIVa₂-ML template (lane 2) or the Rep1, Rep2, Rep3, Rep4, or Rep5 mutant templates (lanes 3–7). End-labeled DNA markers (lane 1) and positions of IVa₂ and *cis*- and *trans*-ML transcripts are indicated as in A. Transcripts of mutant templates initiated at altered sites are indicated by asterisks at the right.

Effects of the Rep substitutions on IVa₂ promoter activity

Our previous analyses of intragenic sequences contributing to the IVa₂ promoter relied on minimal promoters, for example, comprising the HAV-2 IVa₂ sequence from position –9 to position +31 (Chen and Flint, 1992; Chen *et al.*, 1994). To provide a less artificial background, the substitutions described in the previous section were introduced into the IVa₂ promoter in the context of a larger segment of the viral genome, which included the intergenic sequence between the IVa₂ and ML transcription units, the 5' ends of both viral transcription units, and hence the ML promoter. The wild-type and mutant templates were transcribed in whole-cell extracts prepared from uninfected HeLa cells as described, and the quantities of IVa₂ and ML transcripts and the sites at which transcription initiated were then determined by primer extension with appropriate primers (see Materials and Methods). A ML promoter lacking all sequences upstream of position –50 (and therefore the binding site for IVa₂-RF) (see below) linked to a G-less cassette

(Sawadogo and Roeder, 1985a) was used as an internal control. Representative results are shown in Fig. 4.

Although all the Rep substitutions impaired binding of IVa₂-RF to the IVa₂ promoter (Figs. 2 and 3), their effects on IVa₂ transcription were not uniform. Four of the mutations, Rep3, Rep6, Rep7, and Rep8, increased the efficiency of IVa₂ transcription (Fig. 4A). The degree of transcriptional stimulation correlated with reduction in affinity of the cellular protein for its internal binding site in the IVa₂ promoter: the Rep7 mutation, which resulted in a reduction of binding affinity of over 30-fold (Figs. 2 and 3), increased the efficiency of IVa₂ transcription by a factor of close to 5 (Fig. 4A, compare lanes 2 and 5), whereas the Rep3, Rep6, and Rep8 substitutions reduced the affinity of IVa₂-RF for the IVa₂ promoter less than 10-fold (Figs. 2 and 3) and stimulated transcription by factors of some 1.5–3 (Fig. 4A, lanes 3, 4, and 6). The efficiency of transcription from the IVa₂ promoter in HeLa cell extracts is critically dependent on the ratio of extract protein to template DNA concentrations, decreasing sharply as the protein concentration is increased above a narrow range

of optimal values (Leong and Flint, 1984). Consequently, all our studies of the sequences that form the IVa₂ promoter have been performed under conditions that support efficient transcription from this promoter. The use of conditions chosen to reduce such inhibitory effects, as well as the complex organization of the IVa₂ promoter (see Introduction), undoubtedly accounts for the fact that the inverse correlation between binding of IVa₂-RF and efficiency of IVa₂ transcription observed with the set of mutant templates is relative, rather than numerically absolute.

Unexpectedly, these mutations also led to more efficient transcription from the viral ML promoter present *in cis* in the same template (Fig. 4A). This effect was reproducible and also characteristic of the templates carrying additional Rep mutations described in the previous section (e.g., Fig. 4B, compare lanes 2–5). These results indicate that binding of IVa₂-RF to the IVa₂ promoter impairs ML transcription, even though the sequence recognized by this protein lies some 220 bp upstream of the site of initiation of ML transcription (Fig. 1).

Some of the Rep mutations altered the specificity of initiation of IVa₂ transcription, inducing initiation at new sites 7–12 bp downstream of position +1 (Rep2, Rep3, and Rep5; Fig. 4B, lanes 2, 4, 5, and 7) and/or more subtle alterations in the frequency of utilization of initiation sites in the vicinity of those recognized in the wild-type promoter (Rep1, Rep4, and Rep5; Fig. 4B, lanes 2, 3, 6, and 7). All members of this set of mutant templates include substitutions of positions +10 and +11, whereas the mutations that stimulated IVa₂ transcription with no changes in initiation specificity (Rep6, Rep7, and Rep8) did not (Figs. 2 and 4). As these substitutions did not create a sequence common to the mutant templates in the vicinity of the downstream initiation sites (Fig. 2), it appears that the IVa₂ initiator extends to at least position +11 of the transcription unit. It seems unlikely that this component of the promoter extends further downstream by more than a few basepairs, for limited or extensive substitutions between positions +14 and +19 did not alter initiation specificity (Fig. 4A; Chen *et al.*, 1994). In addition, the sequence spanning positions –9 to +13 is sufficient to direct specific initiation of IVa₂ transcription (Carcamo *et al.*, 1991; Chen and Flint, 1992). The substitutions that impinged upon the IVa₂ initiator sequence resulted in at best modest stimulation of transcription (Fig. 4B), even though they inhibited binding of IVa₂-RF to the IVa₂ promoter (Figs. 2 and 3). We therefore conclude that relief from repression of transcription resulting from impaired binding of this cellular protein to the promoter must be offset by unfavorable changes in the initiator or in the downstream sequence required for efficient IVa₂ transcription (Fig. 1).

To provide additional support for the interpretations of the effects of the Rep mutations on IVa₂ transcription given in the previous paragraphs, we sought to separate

the cellular proteins mediating IVa₂ transcription from IVa₂-RF. The latter protein elutes from heparin–agarose at relatively low ionic strength (Chen *et al.*, 1994), whereas the basal RNA polymerase II transcriptional machinery has been reported to require a much higher salt concentration (Dyan and Tjian, 1983). We therefore fractionated HeLa whole-cell-extract proteins on this matrix and examined the ability of fractions recovered to support or alter IVa₂ transcription. The proteins recovered between 0.18 and 1.0 M KCl allowed initiation of transcription from the IVa₂ promoter, the wild-type ML promoter present in the same DNA template, and the truncated ML promoter described above, but at considerably lower efficiencies than observed when whole-cell-extract proteins were supplied (Fig. 5A, compare lanes 2 and 3). The substantial reduction in transcription from all three promoters suggests that essential transcriptional components were diluted below a threshold concentration needed for efficient activity during fractionation. However, we cannot exclude the possibility that proteins specifically required for IVa₂ transcription were not recovered in the 1.0 M KCl heparin–agarose fraction, for utilization of the minor IVa₂ initiation site at position –2 was selectively impaired in reactions containing this fraction (Fig. 5A, lanes 2 and 3). Such different requirements for initiation from the major (+1) and minor (–2) sites are consistent with our previous observation that the –9 to +13 initiator specifies only the major site (Chen and Flint, 1992). Mutations that resulted in more efficient IVa₂ and ML transcription by whole-cell-extract proteins (Fig. 4A) were without significant effect when the templates were transcribed in reactions containing the 1.0 M KCl fraction (e.g., Fig. 5A, lanes 3–6). As this fraction contained no IVa₂-RF activity that could be detected by the electrophoretic mobility shift assay described previously (data not shown), this difference in properties of the mutant templates provides additional support for the conclusion that the stimulation of IVa₂ transcription in whole-cell extracts induced by the mutations is a direct result of impaired binding of the IVa₂-RF to the promoter.

Addition of the IVa₂-RF-containing heparin–agarose fraction to transcription reactions containing basal transcription components further reduced IVa₂ transcription from the wild-type promoter, but inhibited transcription from derivatives carrying substitutions that impaired binding of the cellular protein, such as the Rep6 and Rep7 templates, to a lesser degree (data not shown). Similar results were obtained with the synthetic IVa₂ promoter pIVTnR-TC described previously (Chen *et al.*, 1994) and a mutated version to which IVa₂-RF binds less efficiently. However, the high background observed when the transcriptional machinery was supplied in the 1.0 M KCl heparin–agarose eluate, the consequent low signal:noise ratios, and particularly the very inefficient transcription of the internal control template (e.g., Fig. 5A,

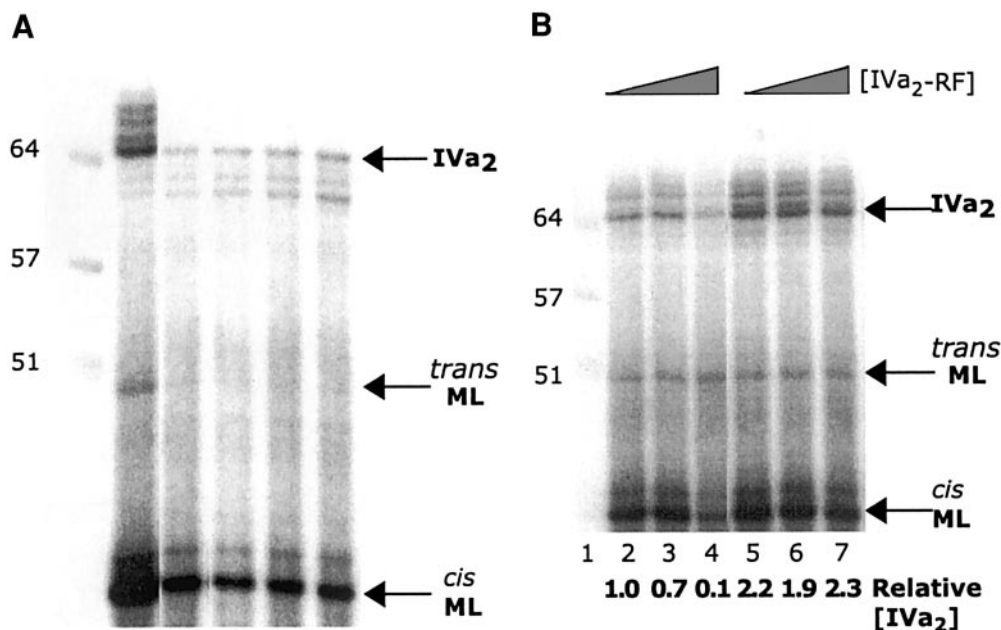


FIG. 5. Transcription from wild-type and mutated *IVa₂* promoters in the absence and presence of *IVa₂*-RF. (A) Transcription reactions contained the internal control pML(*C₂*AT) Δ -50 template and the wild-type pIVa₂-ML template (lanes 2 and 3) or the Rep3, Pep6, or Rep7 mutant derivatives (lanes 4–6, respectively), and 6 mg/ml whole HeLa cell extract protein (lane 2) or 0.38 mg/ml protein of the 1.0 M KCl eluate that contains no *IVa₂*-RF, recovered upon chromatography of such extracts on heparin–agarose (see Materials and Methods) (lanes 3–6). (B) Transcription reactions contained the pML(*C₂*AT) Δ -50 internal control template and the wild-type (lanes 2–4) or Rep6 (lanes 5–7) pIVa₂-ML templates, 6 mg/ml whole-cell extract protein, and 0 (lanes 2 and 5), 0.8 (lanes 3 and 6), or 1.6 (lanes 4 and 7) μ g protein of the heparin–agarose fraction containing *IVa₂*-RF (see Materials and Methods). The concentrations of *IVa₂* transcripts relative to that of those made from the wild-type promoter in the absence of added *IVa₂*-RF-containing fraction, measured as described under Materials and Methods, are indicated below each lane. In both panels, the positions of cDNAs synthesized from *IVa₂*, ML, and internal control (*trans*-ML) transcripts are indicated at the right, and the lengths of DNA markers loaded in lane 1 are listed at the left.

lanes 3–6) precluded reliable quantitative comparisons among the responses of the wild-type and mutated *IVa₂* promoters to the addition of partially purified *IVa₂*-RF. These limitations prompted us to examine the effects of addition of the partially purified protein to transcription reactions containing whole-cell-extract proteins. As the concentration of the *IVa₂*-RF-containing fraction added to such reactions was increased, transcription from the *IVa₂* promoter was reduced by a factor of over 20 (Fig. 5B, lanes 2–4). ML transcription from the same template DNA was similarly inhibited (Fig. 5B, lanes 2–4), to a relative value of 0.04 at the highest concentration of *IVa₂*-RF examined. Such inhibition was a specific consequence of binding of *IVa₂*-RF to the template, for transcription from the internal control template that lacks the binding site for this cellular protein was not altered (Fig. 5B, lanes 2–4 and 5–7). Furthermore, neither *IVa₂* nor ML transcription was impaired when the same concentrations of *IVa₂*-RF were added to reactions containing the Rep6 mutant template (Fig. 5B, lanes 5–7), to which this protein cannot bind efficiently (Figs. 2 and 3). *In toto*, the experiments described in this section establish that binding of the cellular protein dubbed *IVa₂*-RF to its recognition site within this viral transcription unit re-

presses *IVa₂* transcription, as well as transcription from a *cis*-ML promoter.

Effects of HAV-2-infection on the activity of *IVa₂*-RF

The identification of a cellular protein that represses transcription upon binding to the adenoviral *IVa₂* promoter immediately suggests mechanisms accounting for the late-phase-specific transcription of the *IVa₂* gene observed in infected cells (see Introduction). For example, little *IVa₂* transcription is observed in HeLa cell extracts under conditions that support efficient transcription from other promoters (Leong and Flint, 1984) and this adenoviral promoter is inactive in transient expression assays unless linked to the powerful SV40 enhancer (Natarajan and Salzman, 1985). It therefore seems likely that the concentration of *IVa₂*-RF in host cells and its affinity for the binding site in the *IVa₂* transcription unit are sufficient to maintain this promoter in an inactive state during the early phase of infection. The “activation” of *IVa₂* transcription as the infectious cycle enters the late phase would then, in fact, result from relief of such transcriptional repression, for example by virus-induced alterations in the synthesis, stability, or activity of the protein(s) that comprises *IVa₂*-RF. To investigate whether

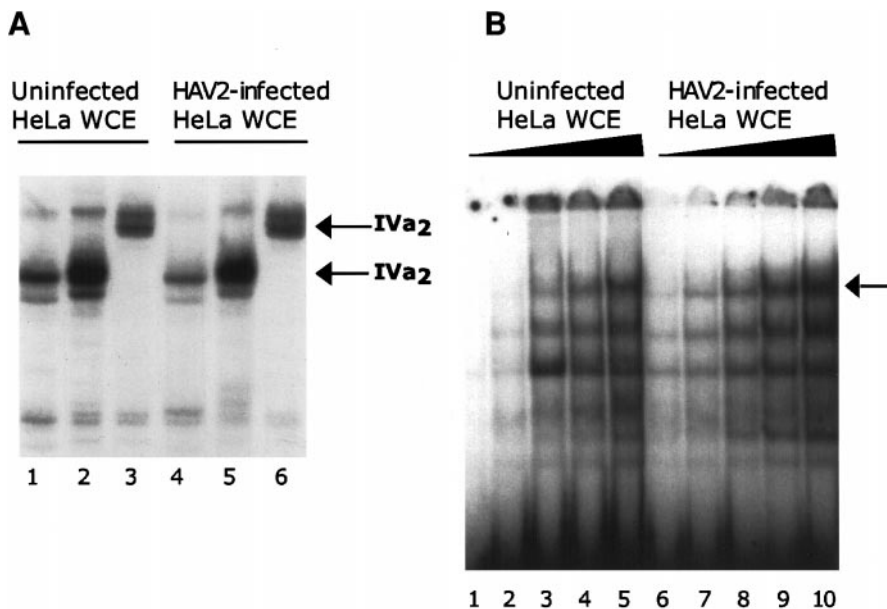


FIG. 6. HAV-2 infection does not alter IVa₂-RF activity. (A) Transcription reactions contained 15 μ g/ml of the synthetic IVa₂ template pIVInr-TC (lanes 1 and 4), its mutated derivative pIVa₂-dsm2 (lanes 2 and 5) to which IVa₂-RF binds poorly, or the pIVa₂-258/+48 template containing both the IVa₂ and the ML promoters (lanes 3 and 6) (see Chen and Flint, 1992) and 5.6 mg/ml whole-cell extract protein from uninfected HeLa cells (lanes 1–3) or from cells harvested 16 h after infection with 15 PFU/cell HAV-2 (lanes 4–6). A primer complementary to a sequence of the neomycin-resistance gene located downstream of the IVa₂ sequences (Chen and Flint, 1992) was used to distinguish IVa₂ transcripts made *in vitro* from endogenous viral RNA present in the infected cell extracts. The positions of the cDNAs copied from the RNAs transcribed *in vitro* are indicated at the right. Templates of the pIVInr-TC series (lanes 1, 2, 4, and 5) fail to direct initiation from the -2 site (Chen and Flint, 1992). (B) DNA-binding reactions were as described under Materials and Methods, except that they contained 1.0 μ g poly(dI \cdot dC) \cdot (dI \cdot dC) and increasing volumes (from 1 to 10 μ l) of whole-cell extracts prepared in parallel from uninfected (lanes 1 to 5) or HAV-2-infected (lanes 6 to 10) HeLa cells. This method of comparison was chosen to attempt to add proteins extracted from equal numbers of infected and uninfected cells to the reactions. The protein concentrations of the uninfected and infected whole-cell extracts added to reactions were 1.23 and 1.34 mg/ml, respectively. The position of the specific complex containing IVa₂-RF is indicated by the arrow at the right.

the production or activity of the protein is indeed modulated as the viral infectious cycle progresses, we compared IVa₂ transcription in extracts prepared from infected or uninfected cells.

Whole-cell extracts were prepared in parallel from uninfected HeLa cells and cells harvested 12–16 h after HAV-2 infection, when IVa₂ transcription is in full swing (see Introduction). The abilities of these extracts to support transcription from a minimal IVa₂ promoter comprising sequences from -9 to $+31$ and from a derivative carrying an intragenic substitution in the IVa₂-RF-binding site (pIVTnR-TC and pIVa₂-dsm2, respectively; Chen and Flint, 1992; Chen *et al.*, 1994) were then compared, using a primer complementary to sequences of the neomycin-resistance gene located downstream of the viral promoter to distinguish transcripts synthesized *in vitro* from IVa₂ RNA recovered in infected cell extracts. No increase in the efficiency of transcription from the wild-type promoter was observed in infected cell extracts (Fig. 6A, compare lanes 1 and 4), the result predicted if the concentration of IVa₂-RF were reduced, or its activity inhibited, in adenovirus-infected cells. Such a lack of effect cannot be ascribed to the minimal nature of the IVa₂ promoter, for a wild-type IVa₂ promoter that retained all upstream sequence to position -258 (including a *cis*-ML

promoter) was also transcribed with indistinguishable efficiencies in uninfected and HAV-2-infected HeLa cell extracts (Fig. 6A, compare lanes 3 and 6). Furthermore, the dsm2 mutation that impairs binding of IVa₂-RF to the IVa₂ promoter (Chen *et al.*, 1994) induced a similar degree of stimulation of IVa₂ transcription in the uninfected and infected cell extracts (Fig. 6A, lanes 2 and 5). These results indicate that HAV-2 infection does not lead to any significant change in the concentration or activity of IVa₂-RF during the period in which the IVa₂ gene is transcribed. To confirm this conclusion, the DNA-binding activity of the cellular protein recovered in uninfected and infected cell extracts was examined directly, using the electrophoretic mobility shift assay described in a previous section. As illustrated in Fig. 6B, no decrease in the DNA-binding activity of IVa₂-RF could be detected by the late phase of infection.

DISCUSSION

Previous studies established that the short sequence spanning the 5' end of the adenoviral IVa₂ transcription unit is functionally complex, including sequences that modulate the efficiency of transcription from an initiator sequence both positively and negatively (Carcamo *et al.*,

1991; Chen and Flint, 1992; Chen *et al.*, 1994). Although the typical set of RNA polymerase II initiation proteins has been shown to be required for transcription from this TATA sequence-lacking promoter (Carcamo *et al.*, 1989), only one cellular protein that binds specifically to the IVa₂ promoter has been identified to date (Chen *et al.*, 1994). This human (HeLa cell) protein, designated IVa₂-RF, contacts specific bases in both strands of the DNA between positions +11 and +27 of the transcription unit. Several lines of evidence reported here establish unequivocally that this protein is a sequence-specific repressor of transcription from this viral late promoter; the effects of the substitution mutations Rep3, Rep6, Rep7, and Rep8 introduced between positions +10 and +20 on the relative affinity with which IVa₂-RF binds to the intragenic promoter sequence correlated inversely with the efficiency of IVa₂ transcription in HeLa cell extracts (Figs. 2, 3, and 4); these same mutations did not induce any stimulation of IVa₂ transcription when transcriptional components were supplied in a fraction lacking IVa₂-RF (Fig. 5A) and increasing the concentration of the latter protein relative to that of the general transcriptional machinery inhibited transcription from the wild-type promoter, but not from a promoter carrying a mutation that impaired its recognition by IVa₂-RF (Fig. 5B).

The binding site for IVa₂-RF defined by methylation interference assays extends into the intragenic sequence required for efficient initiation of transcription from the IVa₂ promoter (Fig. 1). The results presented here indicate that this binding site is also superimposed on the IVa₂ initiator element. As described previously, the altered specificity of initiation of IVa₂ transcription induced by several of the Rep mutations (Fig. 4) indicates that the initiator extends to position +11, a basepair contacted by IVa₂-RF in the wild-type promoter (Chen *et al.*, 1994). The extent to which the repressor-binding site overlaps both the initiator and the downstream stimulatory sequences of the promoter remains to be precisely determined. Nevertheless, the superimposition of the IVa₂-RF binding site on these promoter sequences evident from the data currently available suggests an obvious mechanism for inhibition of transcription by this protein: its binding to the promoter may block access of general initiation proteins and RNA polymerase II to the initiator and downstream promoter sequence (Fig. 1) and thus assembly of preinitiation complexes. Interference with recognition of both of the sequences required for efficient and accurate initiation of IVa₂ transcription would appear to be an effective mechanism of repression. However, the unexpected ability of binding of IVa₂-RF to the IVa₂ promoter to inhibit transcription from the ML promoter (Fig. 4) strongly argues that this protein can interfere with RNA polymerase II transcription by a more active mechanism. The binding site for the repressor is located over 200 bp away from the ML initiation site (Fig. 1), and it does not lie upon any binding sites for

cellular proteins required for efficient ML transcription *in vitro* or in adenovirus-infected cells (Chodosh *et al.*, 1986; Miyamoto *et al.*, 1985; Reach *et al.*, 1990; Sawadogo and Roeder, 1985b). Thus, it appears that IVa₂-RF bound to the IVa₂ promoter must interfere with ML transcription by making contact with one or more of the proteins that mediate or stimulate transcription from this promoter.

As noted previously, the presence of a sequence-specific repressor of IVa₂ transcription in human cells can readily account for the lack of transcription from this promoter during the early phase of infection. We obtained no evidence for inactivation or degradation of this cellular protein by the period in the infectious cycle in which IVa₂ transcription takes place (Fig. 6). We therefore suggest that initiation of IVa₂ transcription in the late phase of the infectious cycle is a direct consequence of the rapid and large increase in the concentration of IVa₂ promoter sequences, following the onset of viral DNA synthesis. During this period, the number of copies of the IVa₂ promoter and IVa₂-RF-binding site increases rapidly, while the concentration and activity of IVa₂-RF do not change significantly (Fig. 6). Thus, above some threshold value determined by the intranuclear concentration of the repressor and its affinity for the binding site in the IVa₂ promoter, the infected cell would contain a greater number of IVa₂ promoters than could be bound by IVa₂-RF. Consequently, some copies would remain repressor-free, allowing IVa₂ transcription to begin (Fig. 7). While this model for the temporal regulation of IVa₂ transcription during adenovirus infection is consistent with the results of the *in vitro* experiments described here, as well as the exquisite sensitivity of IVa₂ transcription to extract protein:DNA ratio (Leong and Flint, 1984), it will be necessary to examine the effects of mutations that impair binding of IVa₂-RF to the promoter upon the kinetics and efficiency of IVa₂ transcription in infected cells. This approach is complicated by both the intricate organization of the IVa₂ promoter and the inclusion of this promoter in the coding sequence for the viral DNA polymerase (Fig. 2), a protein essential for virus reproduction (Roovers *et al.*, 1991). Nevertheless, these *in vitro* studies have identified IVa₂ promoter mutations with appropriate properties, notably the Rep7 mutation, which substantially impairs binding of IVa₂-RF (Figs. 2 and 3) and induces an increase in the efficiency of IVa₂ transcription *in vitro* (Fig. 4), but does not alter the primary sequence of the viral DNA polymerase (Fig. 2).

The adenoviral protein IX (pIX) and E2 late (E2L) promoters are also recognized in infected cells only after the transition into the late phase of infection (see Flint, 1986; Shenk, 1996). As discussed below, two mechanisms mediating temporal regulation of transcription of the pIX gene have been identified, but nothing is known about control of transcription from the E2L promoter, which eventually directs production of the vast majority of E2 transcripts made in the infected cell (Chow *et al.*, 1979).

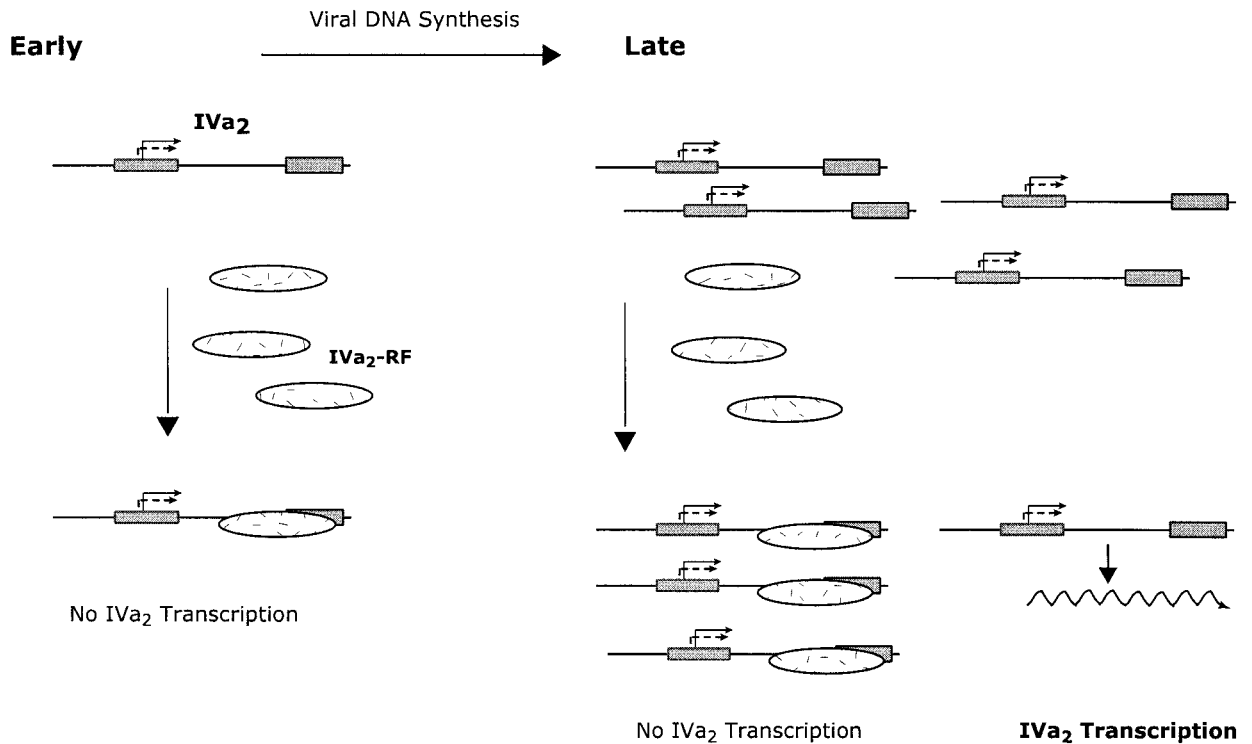


FIG. 7. Model for temporal regulation of IVa₂ transcription, as a result of the viral DNA replication-dependent increase in the intranuclear concentration of this promoter. The IVa₂ transcriptional control region and the cellular IVa₂-RF protein are depicted as in Fig. 1.

A search of the subgroup C adenoviral genome for potential binding sites for IVa₂-RF, using the +11 to +27 sequence of the IVa₂ promoter contacted by this protein (Chen *et al.*, 1994), revealed only one related sequence. This sequence, which is identical at 13/17 positions to the IVa₂-RF-binding site of the IVa₂ transcription unit, lies some 300 bp upstream of the initiation site of the E2L promoter. The presence of so close a match to such a relatively long binding site in the vicinity of this late promoter seems unlikely to be fortuitous. It will therefore be of considerable interest to determine whether IVa₂-RF also contributes to the late-phase-specific activity of the E2L promoter.

The mechanism of viral DNA synthesis-dependent anti-repression proposed for temporal regulation of IVa₂ transcription during the adenoviral infectious cycle (Fig. 7) has a precedent in other viral systems. In particular, Mertz and colleagues have demonstrated that transcription from the most frequently used of the multiple sites of initiation of SV40 late transcription is repressed by binding at multiple sites of a cellular protein (IBP) containing the hERR1 member of the steroid-thyroid receptor superfamily and that mutation of such binding sites leads to stimulation of late transcription during the early phase following transfection of viral genomes into permissive monkey cells (Wiley *et al.*, 1993). A cellular repressor of transcription of the subgroup C adenovirus late protein IX gene has also been identified: the human RBP-2N protein binds specifically to a sequence located immedi-

ately upstream of the TATA sequence of the pIX transcription unit and mutation of this sequence leads to increased production of pIX mRNA during the early, but not the late, phase of infection (Dou *et al.*, 1994). The pIX gene lies entirely within the E1B transcription unit, and relief from promoter occlusion imposed by transcription from the E1B promoter, which is active during the early phase of infection, has also been implicated in the activation of pIX transcription following viral DNA synthesis (Vales and Darnell, 1989). Although the identity of the protein(s) that comprise(s) IVa₂-RF is not yet known, the DNA sequence it recognizes is not related to the binding sites for either of the human IBP or RBP-2N proteins implicated in repression of transcription from other viral late promoters. It therefore appears that the simpler DNA viruses that replicate in primate cells have evolved mechanisms for temporal regulation of transcription of viral late genes that depend on a variety of cellular repressors of transcription.

The repressor titration mechanism for DNA replication-dependent activation of transcription of viral late genes offers the advantage of coupling production of progeny viral genomes in the infected cell with synthesis of the structural proteins from which virions will be assembled. In the case of SV40, such coupling is direct, for the single late transcription unit contains the coding sequence for all three capsid proteins (see Griffin, 1980). The cellular IVa₂-RF protein may also inhibit transcription from the adenoviral ML promoter during the early phase

of infection. However, the ML is as active as other early promoters prior to viral DNA synthesis (Shaw and Ziff, 1980). Thus, during adenovirus infection, such coupling appears to be largely indirect, with production of the IVa₂ protein leading to synthesis of structural proteins via stimulation of ML transcription. The consequences or biological significance of this difference in regulation of the expression of papavo- and adeno-viral late gene products are not yet clear. Identification of the protein(s) comprising IVa₂-RF, and of the second, infected cell-specific protein that cooperates with the IVa₂ protein to induce the dramatic increase in the efficiency of ML transcription characteristic of the late phase of infection (see Introduction) might provide some insight into this issue.

MATERIALS AND METHODS

Cells and virus

HeLa cells were maintained in suspension culture in SMEM (Gibco BRL) containing 5% calf serum, and HAV-2 was propagated in these cells as described (Flint *et al.*, 1975). The titer of virus stocks was determined by plaque assay on HeLa cell monolayers (Williams, 1973).

Construction of mutant IVa₂ templates

The wild-type IVa₂ template, pIVa₂-ML, for the majority of experiments reported here comprised the HAV-2 DNA fragment spanning positions -405 to +180 of the IVa₂ transcription unit in plasmid pUC19. This template therefore also contained the ML promoter, which directs transcription from the opposite strand of the viral genome (see Fig. 1). Precise substitutions were introduced between positions +10 and +20 of the IVa₂ promoter, the sequence that comprises the 5' two-thirds of the previously identified binding site for IVa₂-RF (Fig. 1; Chen *et al.*, 1994), by the unique site elimination method (Deng and Nicholson, 1992). The presence of the desired mutation, but absence of any other change, in the HAV-2 DNA segment, was confirmed by sequencing all mutant DNAs (Sanger *et al.*, 1977). A minimal IVa₂ promoter consisting of the sequence -9 to +31 in pUC19 and a mutant derivative carrying a precise 6-bp substitution within the center of the IVa₂-RF-binding site, in plasmids pIVlnr-TC and pIVa₂-dsm2, respectively (Chen *et al.*, 1994), were used in some experiments. Plasmid DNAs to be used as templates in *in vitro* transcription reactions were prepared and purified as described previously (Chen *et al.*, 1994). The quality and quantity of templates were checked by electrophoresis in 1.4% agarose gels cast and run in 0.098 M Tris-borate buffer, pH 8.3, containing 2 mM EDTA (1× TBE) followed by ethidium bromide staining.

Preparation of cell extracts and separation of IVa₂-RF from proteins necessary for basal transcription

Whole HeLa cell extracts were prepared from actively growing HeLa cells by the modification of the method of Manley *et al.* (1980) described previously (Leong and Flint, 1984). For comparison of the transcriptional activities of uninfected and HAV-2-infected cell extracts, HeLa cells were infected at 15 PFU/ml, or mock-infected, and harvested 12–16 h after infection. Extracts were then prepared in parallel. Protein concentrations were determined by the method of Bradford (1978). For partial purification of IVa₂-RF, whole-cell extracts from 20–30 L freshly harvested HeLa cells were fractionated on Affigel-heparin-agarose (Bio-Rad) by a modification of a protocol described previously (Chen *et al.*, 1994). The column was preequilibrated and loaded in 20 mM HEPES-KOH, pH 7.4, containing 5 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT, 20% glycerol, 1 mM PMSF, and 1 μg/ml each of antipain, pepstatin, and leupeptin (buffer A), and 0.1 M KCl, and IVa₂-RF eluted with 0.18 M KCl in the same buffer. This heparin-agarose fraction was then passed over DEAE-cellulose preequilibrated and loaded in buffer A containing 0.2 M KCl, conditions under which IVa₂-RF did not bind. The flow-through fraction was diluted with an equal volume of buffer A and IVa₂-RF was concentrated by binding to and elution from a small (~2 ml) heparin-agarose column as described above. Following elution with buffer A containing 0.18 M KCl, the original heparin-agarose column was eluted with buffer A containing 1.0 M KCl. This fraction was used as a source of RNA polymerase II and other proteins necessary for transcription from the IVa₂ and ML promoters. All fractions to be used in *in vitro* transcription and DNA-binding assays were dialyzed into buffer A containing 0.1 M KCl, divided into small portions, and stored at -80°C.

Electrophoretic mobility shift assays

The DNA-binding activity of partially purified IVa₂-RF, or IVa₂-RF present in whole-cell extracts, was examined by an electrophoretic mobility shift assay with a ³²P-labeled DNA fragment corresponding to positions +2 to +35 of the HAV-2 IVa₂ transcription unit, as described previously (Chen *et al.*, 1994). Mutant DNAs used as competitors were prepared by annealing complementary, synthetic oligonucleotides containing the mutations that were introduced into IVa₂ transcriptional templates (Kasai *et al.*, 1992). The quantities of specific complex formed in the absence and presence of wild-type and mutant competitor DNAs were measured using a Molecular Dynamics phosphorimager.

In vitro transcription assays

Standard transcription reactions contained 6 mg/ml whole-cell-extract protein, 4.2 μg/μl of the internal con-

trol template pML(C₂AT)Δ-50 comprising the HAV-2 ML promoter from positions -50 to +10 linked to a G-less cassette (Sawadogo and Roeder, 1985a), 8.3 μg/ml template DNA, 12 mM HEPES-KOH, pH 7.9, 67 mM KCl, 6.7 mM MgCl₂, a 0.6 mM concentration each of ATP, CTP, GTP, and UTP, 10% glycerol, and 2 mM DTT in a total volume of 30 μl. Transcription by uninfected cell proteins separated from IVa₂-RF by chromatography on heparin-agarose was assayed in identical fashion, except that reactions contained proteins of the 1.0 M KCl eluate from the column. Reactions were incubated at 30°C for 60 min, and transcripts were purified and analyzed by primer extension as described (Kasai *et al.*, 1992). Primers for detection of IVa₂ and ML transcripts of the pIVa₂-ML templates were complementary to positions +40 to +65 and positions +15 to +40, respectively, of these transcription units. The quantities of cDNAs were determined using a Molecular Dynamics phosphorimager.

ACKNOWLEDGMENTS

We thank H. Chen for performing experiments to compare IVa₂ transcription in uninfected and HAV-2-infected cell extracts, and Jana Kiefer for excellent technical assistance. This work was supported by a grant from the National Institutes of Health.

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